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Paroxysmal Nocturnal Hemoglobinuria: An Acquired Genetic Disease

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder characterized by an intravascular hemolytic anemia. Abnormal blood cells lack a series of glycosylphosphatidylinositol (GPI)-anchored proteins. The lack of GPI-anchored complement regulatory proteins, such as decay-accelerating factor (DAF) and CD59, results in complement-mediated hemolysis and hemoglobinuria. In the affected hematopoietic cells from patients with PNH, the first step in biosynthesis of the GPI anchor is defective. At least four genes are involved in this reaction step, and one of them, an X-linked gene termed *PIG-A,* is mutated in affected cells. The *PIG-A* gene is mutated in all patients with PNH reported to date. Here, we review recent advances in the understanding of the molecular pathogenesis of PNH. Am. J. Hematol. 62:175–182, 1999. © 1999 Wiley-Liss, Inc.

Key words: paroxysmal nocturnal hemoglobinuria (PNH); somatic mutation; glycosylphosphatidylinositol (GPI)-anchored proteins; *PIG-A,* hematopoietic stem cell

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) was first described by Paul Strubing in 1882 as a distinct clinical syndrome with hemoglobinuria after sleep [1]. About 50 years later, Ham demonstrated that the hemoglobinuria is due to abnormal sensitivity of red cells to the hemolytic action of complement [2]. Many hematologists and immunologists were fascinated by this unique feature that host cells are destroyed by complement that is primarily an armor to battle against invading pathogens. PNH was even more interesting because it appeared to be an acquired clonal disorder of hematopoietic stem cells, suggesting that the abnormality had been caused by somatic mutation in the hematopoietic stem cell. PNH lasts for years, sometimes more than 20 years. Thus, the affected stem cells either supply progenies for years or are generated one after another. How affected stem cells dominate hematopoiesis has also been an issue.

In the following sections we summarize recent advances in the understanding of the molecular pathogenesis of PNH and discuss the mechanisms of the predominance of the PNH clone.

CLINICAL MANIFESTATIONS

PNH is an acquired disorder of hematopoietic stem cells; its incidence is estimated at about one case in scores of thousands of persons. Three recent large clinical studies on patients with PNH indicated that it may occur at any age from children as young as 0.8 year to adults up to 82 years and that it is seen most frequently in adults of age 30–50 [3–5]. The clinical manifestation of PNH is complex involving primarily three sets of symptoms: hemolysis with acute exacerbation, cytopenia of varying severity, and a tendency for thrombosis.

Hemolysis and Hemoglobinuria

PNH derives its name from the episode of brownish urine in the morning, although this symptom is found

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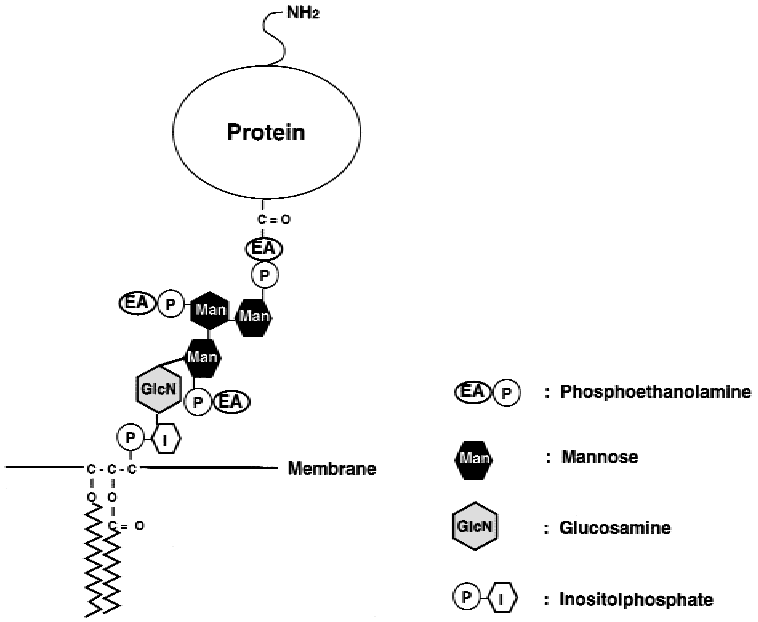

Fig. 1. Structure of mammalian GPI-anchored proteins.

initially in only a quarter of patients. The irregularly recurring exacerbation in hemolysis is often preceded or accompanied by an infection. The extent of hemolysis and the degree of anemia are highly variable from patient to patient.

Cytopenia

Many patients with PNH have evidence of deficient hematopoiesis, and the degree of bone marrow failure varies from subclinical cytopenia to the development of severe aplastic anemia (AA). Also, patients with AA have an increased risk of developing PNH. Recent studies reported that GPI anchor-deficient hematopoietic cell populations emerge in the patients with severe AA at a very high frequency (29–52%) during long-term survival [6–9].

Thromboses

In European-American populations, for unknown reasons, thromboses in PNH almost always involve the venous system, and venous thromboses represent one of the most frequent clinical manifestations of PNH [3–5]. In addition, thrombotic disease is the major cause of death in the patients with PNH [3–5]. On the other hand, the incidence of thrombotic episodes is much lower in Asian than Caucasian populations, and the reason for this is not entirely clear [10,11].

DEFICIENCY OF MULTIPLE GPI-ANCHORED PROTEINS ON CLONAL HEMATOPOIETIC CELLS

The unusual sensitivity of red cells to complement was clarified by the discoveries of a lack of decay accelerating factor (DAF or CD55), an inhibitor of C3 and C5 convertases, and of CD59, an inhibitor of membrane attack complexes. Both are cell surface proteins anchored to the membrane via glycosylphosphatidylinositol (GPI) [reviewed in 12].

Deficiencies in acetylcholinesterase on erythrocytes and alkaline phosphatase on neutrophils in PNH were well known long before the discovery of deficiencies in DAF and CD59. Deficiencies in these and many other GPI-anchored proteins on PNH hematopoietic cells were demonstrated. Since the analysis of genes of GPI-anchored proteins in PNH cells did not show any abnormalities [12], it was proposed that the deficiency of multiple surface proteins must be due to deficient biosynthesis of the GPI anchor.

It should be noted that only a fraction of hematopoietic cells are defective in the surface expression of GPI-anchored proteins. Sizes of abnormal cell fractions vary from patient to patient.

STRUCTURE AND BIOSYNTHESIS OF THE GPI-ANCHORED PROTEINS

The basic structure of the GPI-anchored protein is shown in Fig. 1 [12,13]. Ethanolamine phosphate (EAP), three mannoses (Man), glucosamine (GlcN), and inositol (I) form the core structure, which is conserved in unicellular eukaryotic and mammalian cells [14]. The carboxyl terminus of the protein is covalently linked to the EAP by an amide bond, and EAP is attached to Man at the nonreducing end of glycan. The glycan core consists of a string of three Man molecules and a molecule of GlcN.

Proteins and GPI are synthesized separately in the en-

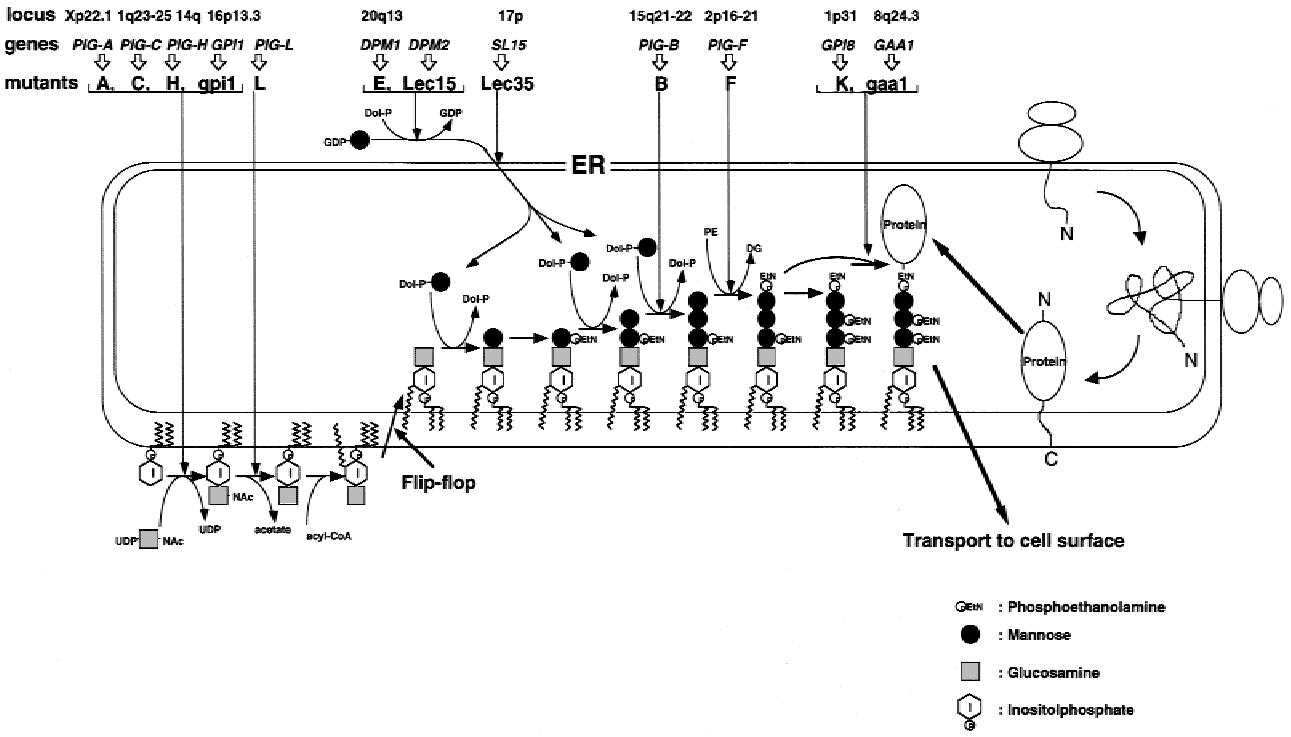

Fig. 2. Schematic representation of biosynthesis of the GPI-anchored protein. Biosynthesis genes, their chromosome loci, and their mutants are shown at corresponding reaction steps. See text for details.

doplasmic reticulum (ER). Proteins are translated with two signal sequences: an amino-terminal signal sequence that directs translocation across the ER membrane and a carboxyl-terminal signal sequence that directs attachment of GPI. The GPI-attachment signal sequence is replaced by a preassembled GPI posttranslationally.

A biosynthetic pathway for the GPI anchor was clarified in trypanosomes, yeasts, and mammalian cells (Fig. 2) [reviewed in 15]. The first step in GPI anchor biosynthesis is the transfer of GlcNAc from UDP-GlcNAc to phosphatidylinositol (PI) to form GlcNAc-PI. This step is mediated by a GlcNAc transferase consisting of at least four proteins: PIG-A, PIG-C, PIG-H, and GPI1 [16]. Genes coding for the first three proteins are defective in GPI-deficient mutant cells of complementation class A, C, and H, respectively. Cells defective in the *GPI1* gene have recently been generated by gene targeting [17]. GlcNAc-PI is then deacetylated to form glucosaminyl-PI (GlcN-PI). This deacetylation step is mediated by a product of the PIG-L gene that is defective in class L mutant cells [18,19]. The inositol residue is then acylated by palmitate. Three Man residues are sequentially added to the glucosamine residue from dolichol-phosphate-mannose (Dol-P-Man). This Man donor is synthesized from dolichol phosphate and GDP-Man by the enzyme Dol-P-Man synthase. Mammalian Dol-P-Man synthase consists of at least two components, DPM1 and DPM2, whose defects are responsible for class E and Lec15 mutants, respectively [20,21]. Lec35 mutant is defective in usage of Dol-P-Man, possibly in its translocation across the ER membrane [22,23]. The three Man should be added by different enzymes. Class B mutant cells are defective in the third Man transfer and a product of the responsible gene *PIG-B* is likely to be an α1–2 Man transferase for this reaction [24]. The final step in the biosynthesis of the core backbone is the transfer of EAP to the third Man, and class F mutant cells are defective in this step [25]. A precursor peptide of the GPI-anchored protein is synthesized with a carboxy terminal signal sequence that directs GPI attachment. A transamidase appears to catalyze replacement of the C-terminal signal peptide and the preformed GPI anchor, generating GPI-anchored protein. At least two proteins, GPI8 and GAA1, are involved in this step [26,27]. The class K mutant is defective in *GPI8* [28] and cells defective in *GAA1* were generated by gene targeting (Ohishi et al., unpublished data). After being processed through the Golgi apparatus, the GPI-anchored proteins appear on the external surface of the cell.

If GPI is not attached due to a lack of biosynthesis, a precursor protein is degraded intracellularly or secreted into the extracellular compartment. Therefore, a lack of GPI anchor biosynthesis causes deficiency of surface expressions of all GPI-anchored proteins.

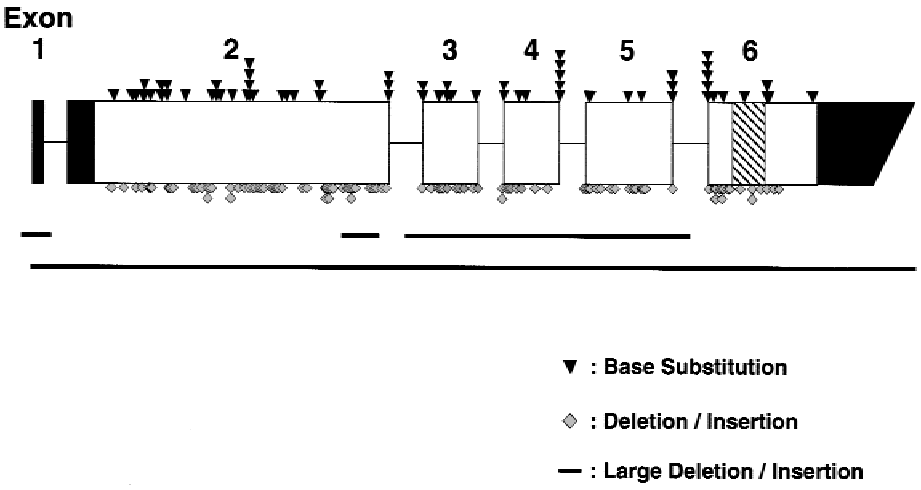

Fig. 3. Structure of the human *PIG-A* gene and locations of somatic mutations found in patients with PNH. Boxes indicate exons. Black areas are noncoding regions. A shaded area indicates a transmembrane domain. Lines connecting exons indicate introns. Base substitutions are indicated above the gene, and deletions, insertions, and other mutations are indicated below.

GENETIC ABNORMALITY OF PNH

Biochemical Defect in PNH

An analysis of GPI anchor synthesis in PNH neutrophils demonstrated that the precursor GPI anchor was not synthesized [29]. Biochemical analyses of several lymphocyte cell lines established from patients with PNH demonstrated that the first step in GPI anchor synthesis is defective [30–33]. At least three different mutant classes (class A, C, and H) defective in this step are known (Fig. 2). Affected PNH cell lines were hybridized with each of the mutants to determine the complementation group of the PNH cells. All of these cell lines complemented classes C and H but not class A mutants on cell fusion [30,34]. These findings led to the notion that the gene defective in PNH must correspond to the gene mutated in the class A mutants.

PIG-A Gene

A cDNA that restores surface expression of GPI-anchored proteins on class A mutants was isolated using the human B lymphoblastoid cell line JY-5 as a recipient for a cDNA library and termed *PIG-A* for phosphatidylinositol glycan class A [35]. *PIG-A* cDNA consists of 4,568 bp and an open reading frame of 1,452 bp and codes for a predicted protein of 484 amino acids, starting at the 86th bp (Fig. 3) [35,36].

The genomic *PIG-A* gene was also isolated using *PIG-A* cDNA as a probe [36,37]. This *PIG-A* gene is at least 17 kb long and has six exons (Fig. 3). Exon 1 (23 bp) is the 5′ untranslated region and exon 2 (777 bp) contains about a half of the coding region. Exon 3 (133 bp), exon 4 (133 bp), and exon 5 (207 bp) contain parts of the coding region and exon 6 (2,316 bp) contains the rest of the coding region and the 3′ untranslated region. The 5′ flanking region (583 bp) has promoter activity [36]. The *PIG-A* gene was localized on the short arm of the X chromosome at Xp22.1 (Fig. 2) [37,38].

As described previously, the *PIG-A* gene product acts in the first step in GPI anchor biosynthesis, and this step is regulated by at least four genes (*PIG-A, PIG-C, PIG-H,* and *GPI-1*) (Fig. 2). A recent study showed that the products of these four genes form a protein complex in the ER membrane and have GlcNAc transferase activity in vitro [16].

Mutations in the *PIG-A* Gene Are Responsible for GPI Anchor Deficiency

An analysis of DNA/RNA from an affected B cell line demonstrated a somatic mutation in the *PIG-A* gene [38]. The same mutation was found in the patient's granulocytes, indicating that the somatic mutation of *PIG-A* occurred in a multipotential hematopoietic stem cell. Moreover, all affected granulocytes from this patient had the same mutation, confirming that they are of clonal origin.

Because *PIG-A* is X-linked, in both male and female somatic cells, only one *PIG-A* allele is active due to the X-chromosome inactivation in the latter. This means that one inactivating somatic mutation would cause GPI anchor deficiency in both males and females.

At least 12 or more genes are involved in the biosynthesis of the GPI-anchor, and some of them have been cloned from human cells (Fig. 2). Mutation of any one of them could result in GPI anchor deficiency; however, the *PIG-A* gene is mutated in all patients with PNH from various countries reported to date (Fig. 3 and Table I) [12,13,39,40]. The most likely explanation as to why *PIG-A* is always responsible is that only the *PIG-A* gene is localized on the X chromosome [37,38,41]. Actually, nine other genes, *PIG-C, PIG-H, GPI1, PIG-B, PIG-F, DPM1, SL15, GP18,* and *GAA1,* have been proven to be autosomal [17,42]. Mutations on both alleles of autosomal genes must occur to cause GPI anchor deficiency, but this event might be rare.

TABLE I. Summary of Somatic Mutations of PIG-A Gene Found in Patients With PNH

I. Type

Type	Number
Base substitution	65
Deletion	
1 nt[a]	48
2 nt	10
3 nt	13
Insertion	
1 nt	20
2 nt	3
3 nt	8
Others	11
Total	178

II. Consequence

Consequence	Number
Frameshift	102
Missense	32
Nonsense	18
Altered Splicing	22
In-frame deletion/insertion	4
Total	178

III. Clonality

PIG-A mutant clones	Number of patients
Mono	121
Oligo	
Two	19
Three	2
Four	2
Total	144

[a]nt = nucleotide.

Abnormalities of the *PIG-A* Gene Found in Patients With PNH

As shown in Fig. 3 and Table I, 179 *PIG-A* mutations have been identified in 146 patients with PNH at latest count [12,13,39,40]. The majority of reported *PIG-A* mutations (133 of 179, 74%) are single-base substitutions, deletions, or insertions, and moreover only 1 or 2 bases are involved in most mutations (146 of 179, 82%). Thus, most of the mutations are small except for four examples: the large deletions (entire gene, 4 kb, and 737 bp) and a large insertion (88 bp). These mutations are widely and heterogeneously distributed in the coding regions and splice sites, and there is no apparent mutation clustering region. The consequence of the majority of these mutations is a frameshift of the coding sequence (102 of 179, 57%).

The missense mutations are interesting because these mutations provide indirect information about critical regions of the PIG-A protein. Some of these missense mutations might be associated with a partial deficiency of GPI-anchored protein. Mutational analysis has suggested that His128, Ser129 and Ser155 are critical residues within the PIG-A protein [43].

Clonality of PNH Cells

PNH has been considered a monoclonal hematopoietic disorder on the basis of the analysis of the X-linked G6PD isozyme [44] and methylation of X-linked genes [45,46] that demonstrated clonality of PNH cells. However, it has been reported that one-third to one-half of patients with PNH have both completely and partially deficient erythrocytes [47]. Some of them also have granulocytes and/or lymphocytes that are partially deficient in the surface expression of GPI-anchored proteins. Mutations of the *PIG-A* gene were demonstrated in those partially deficient cells [48–52]. As described previously, these mutations are usually point mutations that presumably caused a partial loss of PIG-A activity. If indeed partially and completely deficient erythrocytes coexisting in the same patient are independent and distinct mutants of *PIG-A*, then many PNH patients might have more than one mutant clone.

Two independent PNH clones have been documented in nineteen patients (Table I). Moreover, three independent *PIG-A* mutants have been found in two patients, and four mutants have been found in two patients [51,52]. Thus, the presence of four independent mutant clones does not seem to be exceptional. In most cases, one clone is predominant. PNH, therefore, can be regarded as an oligoclonal hematopoietic stem cell disorder.

It is not clear whether *PIG-A* is a hypermutable gene or whether hematopoietic stem cells from patients with PNH have an elevated mutation frequency.

MECHANISM OF CLONAL EXPANSION, A PREREQUISITE FOR CLINICAL MANIFESTATION

Somatic Mutation of *PIG-A* Is Necessary but Not Sufficient for PNH to Occur

For the clinical manifestation of PNH, the domination of hematopoiesis by the *PIG-A* mutant clone is required. Deficiency of GPI due to *PIG-A* mutation would be necessary for clonal expansion because multiple clones of *PIG-A* mutants expand simultaneously. However, two lines of evidence suggest that *PIG-A* mutation alone is not sufficient for the clonal expansion. First, the PNH clone can be found in normal individuals. Araten and colleagues reported that CD59-deficient granulocytes were found in all of nine healthy individuals at a frequency of 22 per million cells [53]. They identified somatic *PIG-A* mutations in six cases. One of them was redetected 164 days later, suggesting that the somatic mutation occurred in a long-living cell. It is not clear

whether this mutated cell in a normal individual is similar to stem cells mutated in the patients with PNH. Nevertheless, this finding supports the notion that the *PIG-A* mutant clone does not expand in a normal bone marrow environment.

The second line of evidence comes from mouse models. Kawagoe and colleagues [54] and Rosti and colleagues [55] disrupted *Piga* gene, a murine homologue of *PIG-A*, in the embryonic stem (ES) cells. Due to the X-chromosomal location of *Piga* and male origin of the ES cells, the *Piga*-disrupted ES cells became defective in the biosynthesis of GPI and the surface expression of GPI-anchored proteins. They generated chimeric mice using the *Piga*-disrupted ES cells and obtained a number of mice which had GPI-negative cells in various blood cell lineages. Percentages of GPI-negative cells did not increase for several months to one year. Therefore, GPI-deficient hematopoietic stem cells did not show immediate expansion.

Murakami and colleagues [56] and Bessler and colleagues [57,58] recently developed a better mouse model in which only the hematopoietic system had *Piga*-deficient cells. Again, GPI-negative cells did not expand for 10–12 months, further confirming that GPI-deficient hematopoietic stem cells do not have an ability to expand.

Candidates for the Second Necessary Factor

If *PIG-A* mutation alone does not cause PNH, what could be the second factor? There are two possibilities. One is that clonal expansion is a result of selection by some pathological mechanism operating in the patients with PNH [59]. A possible mechanism is an immunological selection. This is particularly interesting in view of the frequent association of PNH with AA [6–9] because autoimmunity to hematopoietic stem cells is a very likely cause of acquired AA. If cytotoxic T cells are involved in the autoimmunity, stem cells defective in the surface expression of GPI-anchored proteins may be less sensitive to the cytotoxic cells because the effector–target interaction may be inefficient due to a lack of certain GPI-anchored type of adhesion molecules. Or, when some GPI-anchored protein is an autoreactive antigen, the antigen itself may not be expressed on the cell surface due to a lack of GPI. This might be another mechanism of insensitivity. In any case, selective killing of normal stem cells would result in selective survival and expansion of the GPI-deficient stem cell clone.

The other possibility is that expanded clonal cells have a genetic abnormality in addition to *PIG-A* mutation, and the two in combination may impart an ability to autonomously expand even in a normal bone marrow environment. Iwamoto and colleagues reported that PNH clones in the patients may have such an intrinsic ability [60]. CD34+ hematopoietic progenitor cells from patients with PNH yielded blood cells of three lineages only with the PNH phenotype when transplanted into sublethally irradiated severe combined immunodeficient mice. Human hematopoiesis persisted for more than 10 months and did not always require exogenous human cytokines. In contrast, hematopoiesis of control grafts obtained from healthy volunteers required intense cytokine support. These results may suggest that the PNH clones bearing the *PIG-A* mutation have an intrinsic growth advantage.

It is well known that a few percent of patients with PNH develop acute myelocytic leukemia. In several cases, the leukemic cells were shown to be of PNH origin. It is suggested, therefore, that PNH clones could accumulate multiple genetic abnormalities that ultimately cause the leukemic change.

OTHER POINTS OF INTEREST

PNH lasts for many years. Although cases of spontaneous remission have been reported, most patients have the disease for life [4]. The average life span after diagnosis is somewhere between 10 and 20 years. It is interesting to know whether one clonal mutant stem cell supplies affected blood cells for such a long period of time or mutant clones are generated one after another and maintain the disease. The latter possibility seems more likely because many patients have more than one PNH clone and because even normal individuals have subclinical PNH clones. This suggests that somatic mutation in hematopoietic stem cells occurs from time to time. The subclinical PNH clones would not expand in the normal individuals because they do not have an intrinsic ability to do so whereas in the patients who harbor a pathological environment such clones would eventually expand. If this generation and expansion of PNH clones occurs once in 5–10 years, then the disease would be maintained for life. In line of this, Nafa reported a patient who had PNH and received syngeneic bone marrow transplantation without conditioning [61]. The patient became better but PNH relapsed 10 years later. The authors found that the current and original PNH clones have different *PIG-A* mutations.

It was reported that granulocytes from patients with PNH had a relative resistance to apoptosis and that introduction of the *PIG-A* sequence into a *PIG-A* deficient B-cell line reversed this resistance [62]. The authors concluded that expression of GPI-anchored proteins is critical for the regulation of apoptosis and suggested that its absence leads to clonal dominance. Subsequently, however, different observations were reported from two groups [63,64]. Although granulocytes from patients with PNH have a relative resistance to apoptosis, this resistance was not correlated with the proportion of GPI-negative cells, and the introduction of *PIG-A* cDNA, although it restored expression of GPI-anchored proteins,

did not change the degree of apoptosis [64]. In addition, apoptosis resistance was also observed in a variety of bone marrow failure syndromes including PNH, myelodysplastic syndrome (MDS), and AA [63]. Thus, it does not seem that the *PIG-A* mutation by itself results in impaired apoptosis, although this impaired apoptosis may be instead a feature of bone marrow failure.

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